(12) UK Patent Application (19) GB (11) 2 202 328 (13) A

(43) Application published 21 Sep 1988

(21) Application No 8805681

(22) Date of filing 10 Mar 1988

(30) Priority data
(31) 24604 (32) 11 Mar 1987 (33) US

(71) Applicant
Orion-Yhtyma Oy

(Incorporated in Finland)

P O Box 65, SF-02101 Espoo, Finland

(72) Inventors
Hans Soderlund
Arja Weckman

(74) Agent and/or Address for Service
R G C Jenkins & Co
26 Caxton Street, London, SW1H 0RJ

(51) INT CL[4]
C12Q 1/68

(52) Domestic classification (Edition J):
G1B 104 203 220 223 AC

(56) Documents cited
GB A 2169403 EP A2 0238332 EP A1 0237362
EP A2 0200362
J. Virological Meth. (Nether) (1985) Vol 10
No 2 p 87-98
Biochem. Biophysical Res. Commun US (1987)
Vol 142 No 3 p 710-716
DNA (N.Y.) 1984 Vol 3 No 3 p 269-78

(58) Field of search
G1B
Selected US specifications from IPC sub-class
C12Q

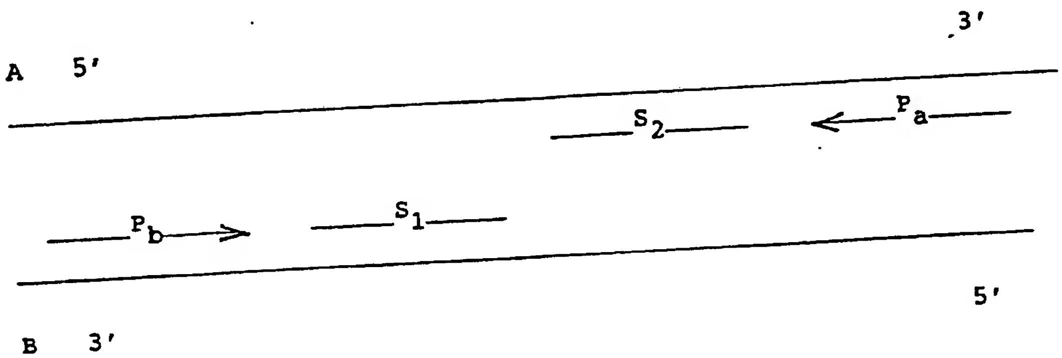
(54) An improved method for assaying of nucleic acids, a reagent combination and a kit therefore

(57) There are provided a rapid and sensitive method for assaying nucleic acids by hybridization in which the detector probes are modified primers incorporated into copies of the target nucleic acid before hybridization reaction, as well as a reagent combination and a kit therefor. There is also provided a method for assaying nucleic acids by hybridization in which the capturing probes are modified primers incorporated into copies of the target nucleic acids before hybridization.

GB 2 202 328 A

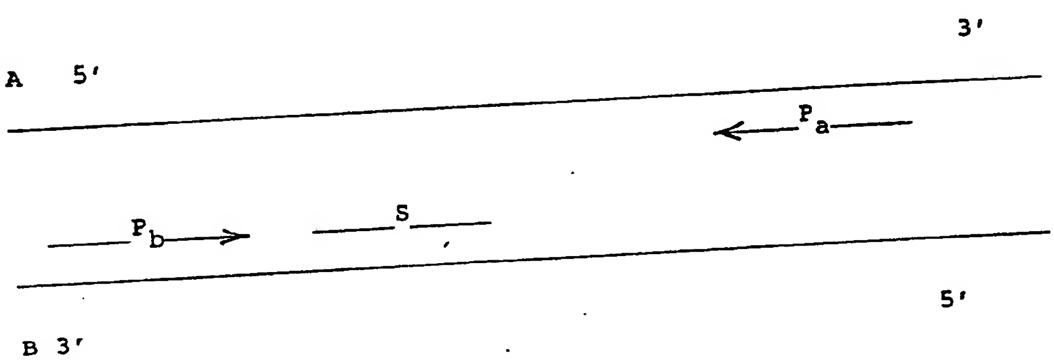
2202328

Fig. 1



P_a: 5'-GAA CAT GTA GTC GGC CAC GG
P_b: 5'-AGC TCT TTC CCG GCC TGG CT
S_1: 5'-ACG TCG CGA GGA CAG CGC CG
S_2: 5'-ATG AGC AGC GCC GCC GCC GT

Fig. 2



P_a: 5'-TGC TCT GGC ATT TGC ATG
P_b: 5'-GAG GCT GAC AGC CCA TTC
S 5'-CAT TTC CGT CGT TCG AGG TGG AGT CAT TGC

2202328

AN IMPROVED METHOD FOR ASSAYING OF NUCLEIC ACIDS, A REAGENT COMBINATION AND A KIT THEREFORE

The present invention relates to a rapid and sensitive method for assaying nucleic acids by means of hybridization techniques, wherein the detector probes are modified primers being incorporated into copies of the target nucleic acid before the hybridization reaction and a reagent combination as well as a kit therefore.

Moreover, the invention relates to a method for assaying nucleic acids by means of hybridization techniques, wherein the capturing probes are modified primers being incorporated into copies of the target nucleic acids before the hybridization reaction and a reagent combination as well as a kit therefore.

In hybridization reactions a labelled oligo- or polynucleotide, i.e. the probe is allowed to base-pair with the nucleic acid target. Various hybridization methods have been used for the detection of nucleic acids. In direct hybridization methods the specimen is either in solution or fixed to a solid carrier. The nucleic acid which is to be identified is demonstrated using one labelled probe.

In US Patent No. 4,486,539 a sandwich hybridization method has been described. In this method two separate probes are used, one being a detector probe labelled and used for detection and the other being a capturing probe immobilized to a solid carrier for the separation of the

target nucleic acid from the reaction mixture.

The method of hybridization in solution is described in British Patent Publication No. 2 169 403. Two different probes both being in the same solution phase are used in this method. The detector probe is labelled with a detectable label and to the capturing probe a moiety having affinity for another component is attached. After the hybridization the hybrid formed between the capturing probe, target nucleic acid and the detector probe, may be separated from the hybridization solution by the aid of the other moiety of the affinity pair.

The enzyme catalyzed polymerization of DNA where the nucleotide sequence of a previously existing nucleic acid strand, i.e. the template is accurately copied into its complementary strand, is well-known in the art and has been described e.g. in Kornberg, DNA replication, W.H. Freeman & Co, San Francisco, pp. 221-225 and 670-679, 1980 and Maniatis et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, p. 122, 1982. This biological multiplication is used in hybridization assays in which the microbe to be detected is cultivated and hence its DNA enriched prior to the test and is described e.g. in Woo, Methods Enzymol. 68, p. 389, 1979 and in US Patent No. 4,358,535. Specific DNA sequences can also be amplified within living cells e.g. by the use of suitable drugs as described by Clewell and Helinski in J. Bacteriol. 110, p. 1135, 1972 and in European Patent Application No. 55 742. A more specific DNA-enrichment is described in in the European Patent Application No. 175 689 in which the target is linked to a plasmid replicon and introduced into a suitable cell. Yet another method is described in the European Patent Application No. 201 184, in which the primer dependence of DNA synthesis is utilized to create an in vitro reaction for the amplification of the target DNA. In the European Patent Application No. 200 362 a method for detecting amplified genes is suggested.

In the hybridization method of the present invention either the detector probes or the capturing probes act as modified primers being incorporated into the copies of the target nucleic acid in a template dependent polymerization process before the hybridization reaction.

In the method of the invention at least one primer is needed and the primers are always modified. If the detector probes act as primers in the polymerization reaction, the primers are provided with at least one suitable, detectable label or at least one specific site whereto at least one suitable, detectable label can be attached.

Alternatively the capturing probes can be used as primers in the polymerization reaction, in which case the primers are provided with at least one suitable moiety of an affinity pair or at least one site whereto at least one suitable moiety of an affinity pair can be attached.

The invention also discloses a reagent combination and a kit comprising in packaged form a multicontainer unit comprising the reagent combination needed for the performance of the test.

By using the detector or capturing probes as primers in a polymerization reaction it is possible to increase the sensitivity of the hybridization reaction by several orders of magnitude compared with methods measuring the target directly. Furthermore, the invention provides a convenient method to perform the hybridization reaction in solution so that the hybrids are easily and rapidly separated from the hybridization solution after the hybridization reaction.

The method of the invention is convenient for diagnosting certain diseases, which are very difficult to diagnose with

conventional methods. Thus the method is especially useful for the identification of cytomegalovirus and the HI- or AIDS-virus.

Fig. 1 represents the base sequence of the modified primers, P_a and P_b, as well the selective probes, S_1 and S_2, used in Example 1, 2 and 3, as well as the relative sites of the modified primers, P_a and P_b, and the selective probes, S_1 and S_2, on the target nucleic acid in question. The long lines, A and B, indicate the two target strands which continue in both directions. The arrowheads on the primers, P_a and P_b, indicate the direction in which they are elongated in the polymerization process.

Fig. 2 represents the base sequence of the modified primers, P_a and P_b, and the selective probe, S used in Example 4, as well as the relative sites of the modified primers, P_a and P_b and the selective probe S on the target nucleic acid in question. Line A indicates the RNA and its identical DNA copies and line B shows the complementary DNA copies. The arrowheads on the primers P_a and P_b indicate the direction in which they are elongated in the polymerization process.

The probes used in the method are oligo- or polynucleotides. The probes can be prepared synthetically or semi-synthetically, which are the preferred mode to prepare probes, which will act as primers, too. It is also quite possible to prepare the probes by recombinant techniques, or from nucleic acids isolated directly from nature. A probe may be bound to a suitable vector. It may contain vector parts or be completely devoid of vector

parts. Actually a multitude of suitable primers and probes, which can be used, are commercially available.

In one of the methods of the present invention the detector probes are oligonucleotides or polynucleotides, which can be bound to the target nucleic acid by base-pairing and which can act as primers for a template dependent nucleic acid synthesizing enzyme. It is essential that the detector primers are provided with at lest one suitable, detectable label or at least one specific site whereto at least one suitable detectable label can be attached.

Various radioactive isotopes or radioactively labelled compounds may be used as labels. The label substance may also be fluorescent, luminescent, light emitting, enzymatically or immunologically demonstrable etc. Labels based on the affinity of biotin and avidin or streptavidin, lanthanide chelates, ferritin and heme compounds, and immunologically demonstrable haptens such as AAF and AIF (acetoxyacetylfluorene derivatives) can be mentioned as examples. Identification by the aid of mediators, for example proteins, is also possible.

The method according to the invention is not dependent on the label used. All currently known label substances suitable for nucleic acid hybridization can be freely applied to the method. It is, however, essential that if the detector probes act as primers, the label is selected from a group of labels, which will not disturb the function of the primer. The label has to be attached to the detector primer in such a way that the nucleic acid polymerizing enzyme still can recognize it as a primer.

In the other method of the present invention the capturing probes are oligonucleotides or polynucleotides which can be bound to the target nucleic acid by base-pairing and which can act as primers for a template dependent nucleic acid synthesizing enzyme. It is essential that the capturing primers are provided with at least one suitable moiety of an affinity pair or at least one specific site whereto at least one suitable moiety of an affinity pair can be attached. It is also possible to attach the moiety or moieties of the affinity pair through a mediator to the capturing primer. The only conditions are that it is possible to separate the hybrid from the hybridization solution by the aid of the affinity pair and that the primer function is not harmed.

The moiety of the affinity pair is a component having affinity for another component. For example, biotin - avidin or streptavidin, a heavy metal derivative - a thiogroup, various homopolynucleotides such as poly dG - poly dC, poly dA - poly dT, and poly dA - poly U, are such affinity pairs. But also other component pairs can be used, provided that they have affinity strong enough to allow the specific binding of the modified capturing primers having been incorporated to the copies of the target nucleic acid to the solid carrier. Suitable affinity pairs are found among ligands and conjugates used in immunological methods.

In one of the methods of the present invention, wherein the detector probes act as primers, a selective capturing probe is required to allow the selective separation of the copies of the target nucleic acid wherein the modified primers have been incorporated. It is essential that the capturing probes are sufficiently homologous to the target

nucleic acid to allow their specific hybridization with the copies of the target nucleic acid and thereby selective separation and detection of the detector primers having been incorporated into the copies of the target nucleic acid.

In the other method of the present invention, wherein the capturing probes act as modified primers, a selective detector probe is required to allow the detection of the the copies of the target nucleic acids wherein the modified primers have been incorporated. It is essential that the detector probe is sufficiently homologous to the target nucleic acid to hybridize specifically and thereby to identify the target nucleic acid selectively. The detector probes can be provided with any suitable, detectable labels for example with those mentioned above.

The present invention relates to a reagent combination comprising at least one modified primer, provided with at least one suitable, detectable label or at least one specific site whereto at least one suitable, detectable label can be attached and at least one selective capturing probe provided with at least one moiety of an affinity pair or at least one specific site whereto at least one moiety of an affinity pair can be attached.

The present invention relates also to a reagent combination comprising at least one modified primer provided with at least one suitable moiety of an affinity pair or at least one specific site whereto at least one suitable moiety of

an affinity pair can be attached and at least one selective detector probe provided with at least one suitable, detectable label or at least one specific site whereto at least one suitable, detectable label can be attached.

The present invention also discloses a convenient kit for assaying nucleic acids. The kit comprises in packaged form a multicontainer unit in which one of the reagent combinations mentioned above is combined with at least one of the following reactants or facilities needed in the test i.e. optionally a container comprising at least one template dependent polymerization agent, optionally a container with the four deoxynucleoside triphosphates, optionally a suitable facility for the polymerization and the hybridization process, optionally a suitable facility for the separation of the copies of the target nucleic acids and optionally a suitable facility for assaying the label. The preferred facilities and reactants are described in more detail in the following part of the specification.

The preferred method of the present invention starts by adding at least two modified primers, both primers being either detector or capturing primers, to a denaturated sample solution. The modified primers will each anneal to their complementary strand of the target nucleic acid, i.e. the template and upon addition of a template dependent nucleic acid synthesizing enzyme the primers will be elongated. The process proceeds efficiently in vitro creating new nucleic acid strands which may be several thousand bases in length, provided the conditions are suitable.

By using an excess of modified primers the process may be repeated to create complementary copies to the newly

synthesized strands, which thus are identical copies of the first template. By repeating this process a cascade reaction is initiated whereby the target nucleic acid is multiplied. The process may be repeated as many times as desired, to obtain the desired detection sensitivity. In cases where the concentration of target nucleic acid is not extremely low one multiplication is sufficient to make the target nucleic acid detectable.

It is also possible to use only one modified primer in the method of the invention. In this case the multiplication is, however, not so efficient as by using at least two primers because the reaction is not a cascade type reaction.

Both DNA and RNA can be determined by the method of the present invention. However, if the target nucleic acid is RNA it is most convenient first to copy the RNA to the corresponding cDNA by reverse transcriptase enzyme, whereafter the process continues as described above.

After the modified primers are incorporated into the copies of the target nucleic acids, a suitable selective probe recognizing the target sequence and its copies is added to the reaction mixture and the hybridization reaction is performed under conditions suitable for the respective hybridization process chosen.

In the hybridization reaction, depending on the choice of modified primers, either a selective capturing probe or a selective detector probe is allowed to hybridize with the copies of the target nucleic acid now present in multifolded amounts compared to the amount of the target nucleic acid in the original situation.

If the original sample contained the target sequence the added selective probe will hybridize to newly synthetized copies of the target nucleic acid. A hybrid is formed

between the copy molecule wherein the modiefied primer has been incorporated and the selective probe. The hybrids formed are according to the present invention, conveniently separated from the hybridization solution by the aid of the moiety of the affinity pair, which is attached, either on the capturing primer or on the selective capturing probe. During fractionation these capturing moiety containing hybrids adhere to a solid carrier by the aid the other moiety of the affinity pair and the amount of selective detector probe or detector primer adhering to the carrier can be measured by conventional methods directly from the carrier or after elution from the eluate. The amount of label is a measure of the amount of target nucleic acid.

Before the fractionation, the solution is diluted, when necessary, to render the conditions advantageous for the affinity pair. Thereafter the solution is contacted with the solid carrier. The carrier in question may be for instance an affinity chromatography column, a filter, a plastic surface or a glass surface. Convenient facilities for performing the separation are different types of microtiter plates, dipstick systems or magnetic particles, but it is quite possible to perform the separation in test tubes and on beads etc.

The carrier material of the affinity column may be natural or synthetic polymer, for instance, cellulose, polyacrylamide, polystyrene, dextran or agarose. These materials can also be used as suspensions in a test tube. It is also advantageous to use test tubes having the other moiety of an affinity pair fixed to its inner surface. It is a prerequisite for the material selected that it is possible to fix to it a component having affinity to the moiety of the affinity pair which is attached to the capturing primer or the selective capturing probe.

It is not necessary to attach the moiety or moieties of the affinity pair to the capturing primer at the beginning

of the polymerization. Neither is it necessary to attach the detectable label to the detector primer before the polymerization. These may also be added after the polymerization process to the modified primer having been incorporated into the copies of the target nucleic acid. For example, when the detectable label is sensitive to the hybridization conditions it may be added first after the hybridization of the selective capturing probe with the copies of the target nucleic acid.

If the detector probes act as modified primers being incorporated into the copies of the target nucleic acid, the hybrid can be separated from the reaction mixture by the aid of selective capturing probes immobilized on solid carriers. In this method which has also been described in the European Patent Application No 237362 the rate limiting step is created when the target nucleic acid and its copies, wherein detector primers have been incorporated, must hybridize with the selective capturing probe that is immobilized on a solid carrier. Therefore, the hybridization in solution is a more preferred method of the invention than this. However, if the method is carried out by using an immobilized capturing probe, the hybrid formed on the solid carrier is washed and the amount of the label on the carrier is measured by conventional methods.

The principle of the test is demonstrated in the following examples.

Example 1

Detection of cytomegalovirus DNA by using detector probes as modified primers

In this model experiment the target was a recombinant plasmid (pBR322/CMV HindIII L) which harboured a 12.3 kb fragment of the cytomegalovirus (CMV, AD 169, ATCC VR-538) genome. The two detector primers (P_a, P_b, Fig. 1) used were

20 nucleotides long and synthesized with standard methods on an automated synthesizer. They corresponded to two regions on the CMV-specific insert which were 111 nucleotides apart. Two selective capturing probes (S_1, S_2, Fig. 1) recognized regions on each of the two strands between the two detector primers. The detector primers P_a and P_b were labelled with ^{32}P at their 5' ends to a specific activity of 4×10^9 CPM/μg using the well known reaction with polynucleotide kinase and gamma-^{32}P-ATP (Maniatis et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, 1982).

Biotinylated nucleotides were added to the 3' ends of the capturing probes using bio 11-dUTP (BRL) and terminal transferase (Promega Biotech.) as described by Riley et al., DNA, 5 (4), pp. 333-337, 1986. The target plasmid was linearized by cleavage with the restriction enzyme EcoRI. DNA polymerase, Klenow fragment was purchased from Boehringer-Mannheim and streptavidin agarose from BRL.

Using these reagents the following experiment was performed.

Four different reactions were assembled containing 0, 10^4, 10^6 and 10^8 molecules (corresponding to 0, 2×10^{-20}, 2×10^{-18} and 2×10^{-16} moles) respectively of the target plasmid. In addition all four reactions contained in a total volume of 50 μl: 2 pmol each of the two primers, 0,5 mM of each of the four deoxynucleoside triphosphates (i.e. dATP, dCTP, dGTP and dTTP), 10 mm Tris-Cl (pH 7,5), 10 mM $MgCl_2$, 50 mM NaCl and 10 mM dithiothreitol. The mixture was heated to 100°C for 2 min, then incubated for 5 min at 37°C, whereafter 1 μl (equalling 1 unit) of DNA-polymerase was added. Then the mixture was again incubated for 10 min at 37°C. The boiling followed by annealing of the detector primers and an incubation with added DNA polymerase at 37°C constitutes a DNA synthesizing cycle.

In this experiment the cycle was either performed only once or repeated 5 or 15 times. After the last cycle the sample was again heated to 100^{o}C whereafter 10 pmole of the selective capturing probe was added together with NaCl (0,9 M), EDTA (20 mM), sodium phosphate (pH 7,5; 20 mM) and sodium dodecyl sulphate (0.1 %). The volume increased to 100 μl and the concentrations given are as final concentrations. The mixture was then incubated at 50^{o}C for 1 h. After this hybridization reaction 200 μl of a 25 % suspension of streptavidin-agarose in 1 M NaCl, 20 mM sodium phosphate (pH 7,5), 1 mM EDTA was added. Biotinylated molecules were allowed to bind to the streptavidin-agarose for 15 min at 37^{o}C in a rotating mixer. The agarose was collected by brief centrifugation and the supernatant removed by aspiration. The agarose was then washed once in the buffered 1 M NaCl and twice in a solution containing 150 mM NaCl, 15 mM sodium citrate and 0.2 % sodium dodecyl sulphate (pH 8) at 37^{o}C. The radioactivity of the agarose to which the formed hybrids were bound was then determined in a radioactivity counter. The harvesting and washing procedure for DNA hybrids containing a biotinylated marker are previously known procedures described e.g. in British Patent Publication No. 2 169 403.

The results of the experiment are shown in Table 1. It is seen that one cycle of DNA synthesis incorporates enough radioactivity for detection only if high target concentrations are present, but that even the very low target amount is detectable after 15 cycles. With high amount of target and 15 cycles the amount of detector primer became limiting.

Table 1

Amount of target (moles)	^{32}P-activity in collected hybrids [a)] (CPM above background) [b)]			
	1	5	15	No. of cycles
0	0	0	0	
2×10^{-20}	ND	ND	650	
2×10^{-18}	ND	300	11000	
2×10^{-16}	700	13000	36000	

a) Mean of two determinations
b) ND - not detectable (less radioactivity than 2 times mean background activity)

Example 2

Determination of cytomegalovirus DNA by using capturing probes as modified primers.

In this example the capturing probes act as primers. The reagents used were the same as in Example 1 with the following exceptions: The capturing primers (P_a, P_b, Fig. 1) were not labelled with ^{32}P but their 5' ends were instead modified to contain a biotin residue. This chemical modification was done using known methods described by Chollet and Kawashima, Nucleic Acids Research, 13, pp. 1529-1541, 1985. The two selective probes (S_1 and S_2, Fig. 1) were in this case labelled in their 5' ends to act as detector probes. Their specific activities were approximately 2×10^9 and $2{,}5 \times 10^9$ cpm/μg respectively.

The reaction mixtures were assembled as described in Example 1. The biotinylated capturing primers were, however, added in 10 fold amounts, i.e. 20 pmol each per reaction. 1, 5 or 15 cycles were performed as described

whereafter the samples were heated to 100°C and 0,5 pmol each of the ^{32}P-labelled probes S_1 and S_2 were added. The hybridization was carried out in the same conditions as described in Example 1.

The hybrids were then collected on streptavidin-agarose, washed and counted for ^{32}P-activity, as in Example 1. The result is shown in Table 2.

Table 2

Amount of target (moles)	^{32}P-acitivity in collected hybrids a) (CPM above background) b)			
	1	5	15	No. of cycles
0	0	0	0	
2×10^{-20}	ND	ND	800	
2×10^{-18}	ND	400	13000	
2×10^{-16}	300	11000	54000	

a) Mean of two determinations
b) ND - Not detectable (cf ex 1)

Example 3

Detection of cytomegalovirus DNA from clinical samples by using capturing probes as modified primers.

In this example the applicability of the method for the study of clinical samples is demonstrated by detecting CMV from the urine of an infant known to suffer from cytomegalovirus infection. The urine from a healthy child was included as a control. Both samples were 10 ml of urine from which the total DNA was isolated as described in Virtanen et al., J. Clin. Microbiol., 20 (6), pp. 1083-1088, 1984. The DNAs, dissolved into 20 µl H_2O were

used as target in reactions which otherwise were performed as described in Example 2. After 10 cycles of DNA-synthesis the labelled selective probe was added to the sample, allowed to hybridize, and the hybrids collected. The DNA from the urine of the patient showed a clearly elevated radioactivity in hybrids while that from the healthy person showed bakckground radioactivity only. The actual cpm-values were 2300 and 240 respectively.

Example 4

Detection of Semiliki Forest virus RNA by using capturing probes as modified primers.

Example 4 is to demonstrate that the method described also can be used for the detection of RNA. The model used was the RNA from Semliki Forest virus (SFV).

The reagents used were two 5' biotinylated capturing primers (Fig. 2) (prepared as described in Example 2), a single 5' ^{32}P-labelled selective detector probe (prepared as described in Example 1), reverse transcriptase (Promega Biotech) and DNA polymerase, Klenow fragment (Boehringer Mannheim).

The first step in the detection of the SFV-RNA was to synthesize a cDNA copy. The 20 μl reaction mixture contained 10 mM tris-Cl (pH 8.3), 50 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol 0,5 mM each of the four deoxynucleoside triphosphates, 0,5 μg t-RNA, 10 pg of SFV-RNA, 10 pmol of capturing primer P_a and 100U reverse transcriptase. This mixture was incubated at 37°C for 15 min. Then the mixture was heated to 100°C for 5 min and cooled to ambient temperature. Thereafter 50 μl of a solution containing 10 mM Tris-Cl (pH 7,4), 50 mM NaCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 10 pmol of the capturing primer P_b and 0,5 mM of each of the 4 deoxynucleosidetriphosphates was added. The temperature was elevated to 37°C

and after 5 min 1 U of DNA-polymerase was added. After an additional 10 min incubation the reaction mixture was incubated at 100°C for 5 min, the mixture was cooled to 37°C and 5 cycles of DNA synthesis was performed. After a final denaturation step 0.1 pmol (1,2 x 10^6 cpm) of the selective detector probe was added in 80 µl 1 M NaCl, 50 mM EDTA, 50 mM sodiumphospate (pH 7,5) and 0.1 % sodium dodecyl sulphate. The solution was then incubated for 2 h at 55°C whereafter the hybrids were collected and washed as described in Example 1.

As a negative control for the reactions an identical sample was given the same treatment except for that no reverse transriptase was added. The sample in which the RNA was converted to cDNA with reverse transcriptase yielded 420 cpm ^{32}P-activity in captured hybrids, while the negative control yielded 50 cpm.

Example 5

Comparison of different modes of detection of multiplied DNA

In this example three different modes of detection of multiplied DNA were compared. The reagents and the multiplication process were as described in Examples 1 and 2 except that the selective capturing or detector probes were M13 clones recognizing about 100 nucleotides between the primers.

The M13 clones were obtained by subcloning of a HaeIII restriction fragment of the recombinant plasmid pBR322/CMV HindIII L into the phage vector M13mp10 using standard techniques. The M13 clones to be added as selective capturing probes were modified with biotin using photoprobe™ biotin (Vector Laboratories). The M13 clones to be used as selective detector probes were labelled with ^{32}P-dCTP using DNA polymerase I (the Klenow fragment) and

primer extension (Hu and Messing, Gene 17, pp. 271-277, 1982) to a specific activity of 2 x 10^8 cpm/μg.

The multiplication of 3 x 10^5 molecules (0,5 x 10^{-18} moles) of the linearized pBR322/CMV HindIII L plasmid was carried out with 10 cycles. For detection according to Modes 1 and 2, 2 pmoles each of the ^{32}P labelled detector primers P_a and P_b were employed and the multiplication process was done as described in Example 1. For detection according to Mode 3, 25 pmoles of the biotinylated capturing primers were used in the multiplication procedure and the reaction was carried out as described in Example 2.

Mode 1 of detection - Selective capturing probe used for collection of hybrids formed in solution with the copies of the target nucleic acid

In this mode of detection biotinylated selective M13 capturing probes were used to collect the multiplied DNA fragments. After the last multiplication cycle the sample mixture was heated to 100°C and 2 x 10^9 molecules each of the biotinylated selective capturing probes were added together with NaCl (0,6 M), EDTA (5 mM), sodium phosphate (20 mM) and SDS (0,1 %). The volume increased to 100 μl and the final concentrations are given. The mixture was incubated at 65 °C for 2 hours. The hybrids formed were collected on streptavidin-agarose as described in Example 1, except that two additional 1 minute washes at 50°C with 15 mM NaCl, 1,5 mM sodium citrate, 0,2 % SDS were done. The radioactivity of the collected hybrids was measured.

Mode 2 of detection - Selective capturing probe immobilized before its hybridization with the copies of the target nucleic acid

In this case immobilized selective M13 probes were used to capture the multiplied DNA. After the last cycle of multiplication the samples were heated to 100°C.

NaCl (0,6 M), sodium citrate (60 mM), Ficoll™ (0,02 %), polyvinylpyrrolidone (0,02 %), bovine serum albumin (0,02 %) and denatured herring sperm DNA (0,2 mg/ml) were added to give the indicated concentrations in a final volume of 100 μl. A nitrocellulose filter disc, to which 5×10^{10} molecules each of the selective probes had been immobilized according to a previously described procedure (US 4,486,539) was added to each sample. The mixture was incubated with the filters at 65°C for 2 hours or 18 hours. After the hybridization reaction the filters were washed twice for 20 min at 50°C with 15 mM NaCl, 1,5 mM sodium citrate and 0,2 % SDS and the radioactivity bound to the filters was measured.

Mode 3 of detection - Selective detector probe used for detection

Here ^{32}P labelled selective M13 detector probes were used for the detection of the multiplied DNA and the hybrids were collected by the aid of the capturing primers biotinylated at the 5' ends as described in Example 2. The multiplied samples were heated to 100°C and 2×10^8 molecules (2×10^5 cpm) each of the ^{32}P labelled selective probes were added together with salts as described for Mode 1. Hybridization and collection of the formed hybrids was done as for Mode 1.

A comparison of the results obtained by the three modes of detection is shown in Table 3.

Modes 1 and 3 have the advantage of the faster rate of hybridization in solution compared to the filter hybridization in Mode 2. The highest ^{32}P activity is obtained by Mode 3 because the selective detector probe contains multiple ^{32}P atoms per molecule.

Table 3

Mode	Primers	Selective M13 probe	Hybridiz. time (hours)	32P activity in hybrids (cpm) a)
1	^{32}P-labelled detector	biotinylated capturing	2	870
2	^{32}P-labelled detector	immobilized capturing	2 18	43 920
3	biotinylated capturing	^{32}P-labelled detector	2	7800

a) Mean of three determinations (cpm above background)

Example 6

Multiplication of cytomegalovirus DNA using biotinylated detector primers and indirect detection with streptavidin-horseradish peroxidase

In this example multiplification of the CMV specific plasmid (pBR322/CMV HindIII L) was done using the biotinylated primers P_a and P_b described in Example 2 as detector primers. The M13 clones described in Example 5 modified with sulfone groups were used as selective capturing probes. The formed hybrids were collected in microtitration wells coated with antibodies recognizing sulfone modified DNA. The final detection of the formed hybrids was done with a streptavidin-horseradish peroxidase conjugate, which detects the biotin moieties of the primers.

The M13 clones were modified by a sulfonation reaction using reagents from and the procedure recommended by

Orgenics Ltd (Yavne, Israel). Polystyrene microtitration wells (Nunc, Denmark) were coated with 10 µg/ml of IgG purified from a monoclonal antibody against sulfone modified DNA (Orgenics Ltd) in 10 mM sodium carbonate buffer (pH 9,6) over night at 4°C.

A reaction mixture containing 3×10^5 molecules of the linearized pBR322/CMV HindIII L plasmids or controls without the plasmid and 25 pmoles each of the biotinylated primers P_a and P_b were processed for 10 multiplication cycles at the conditions described in Example 1. The samples were heated to 100°C, whereafter 2×10^9 molecules each of the sulfonated selective capturing probes were added together with reagents as specified for Mode 1 of detection in Example 5.

The mixture was incubated at 65°C for 2 hours, diluted with 100 µl of 0,2 % Tween 20 and transferred to the coated microtitration wells. The hybrids were allowed to bind to the wells for 2 hours at 37°C. The reaction mixture was discarded and the wells were washed three times with 0,1 % Tween 20 in 0,15 M sodium chloride, 20 mM sodium phosphate (pH 7,5) (PBS). 200 µl of a streptavidin-horseradish peroxidase conjugate (Amersham, UK) diluted 1:2000 in a solution of 1 % bovine serum albumin, 0,1 % Tween 20 in PBS was added and the wells were incubated for 45 min at 37°C. After four washes as above, 200 µl of a substrate solution consisting of 0,46 mg/ml O-phenylene-diamine, 0,01 % H_2O_2 in 0,1 M sodium acetate buffer (pH 5,0), was added. After 15 min at 22°C the reaction was stopped by the addition of 50 µl of 2N H_2SO_4 and the absorbance of the coloured product was measured with a spectrophotometer at 492 nm. The collection and detection procedures have been previously described by Syvänen et al. (Nucleic Acids Res, 14, pp. 5037-5048, 1986).

The results of the experiment are shown in Table 4. 3×10^5 molecules ($0,5 \times 10^{-18}$ moles) of the target plasmid

were clearly detected after 10 cycles of multiplication.

Table 4

Amount of target (moles)	Absorbance at 492 nm a)
$0,5 \times 10^{-18}$	0,348
0	0,120

a) Mean of triplicate assays

CLAIMS

1. A method for assaying nucleic acids by hybridization c h a r a c t e r i z e d in that the capturing probes act as modified primers being incorporated into the copies of the target nucleic acid, the presence of which is then detected by at least one selective detector probe.

2. The method as claimed in claim 1 c h a r a c t e r i z e d in that it comprises:
(a) providing at least one primer of the target nucleic acid with at least one moiety of an affinity pair or at least one specific site whereto at least one moiety of an affinity pair can be attached;
(b) allowing said capturing primer or primers to react with the single-stranded target nucleic acid under conditions suitable for a template dependent polymerization reaction;
(c) allowing the single-stranded copies of target nucleic acid wherein the capturing primers have been incorporated to hybridize with a detector probe capable of selectively hybridizing with the target nucleic acid under conditions suitable for a hybridization reaction;
(d) separating the copies of the target nucleic acid wherein the capturing primers have been incorporated by the aid of the other moiety of the affinity pair;
(e) detecting the presence of the selective detector probes having hybridized with the copies of the target nucleic acid.

3. A method for assaying nucleic acids by hybridization, wherein the detector probes act as modified primers being incorporated into the copies of the target nucleic acids c h a r a c t e r i z e d in that the copies of the target nucleic acids wherein the modified primers have been incorporated, are first hybridized with at least one selective capturing probe and the hybrid formed is then separated by the aid of said capturing probe whereafter the presence of the hybrid is detected.

4. The method as claimed in claim 3 c h a r a c t e r i-z e d in that it comprises:
(a) providing at least one primer of the target nucleic acid with at least one detectable label or at least one specific site whereto at least one detectable label can be attached;
(b) allowing said detector primer or primers to react with the single-stranded target nucleic acid under conditions suitable for a template dependent polymerization reaction;
(c) allowing the single-stranded copies of the target nucleic acid wherein the detector primers have been incorporated to hybridize with a capturing probe capable of selectively hybridizing with the target nucleic acid under conditions suitable for a hybridization reaction;
(d) separating the copies of the target nucleic acid wherein the detector primers have been incorporated by the aid of the selective capturing probe;
(e) detecting the presence of the copies of target nucleic acids.

5. The method as claimed in claim 3 or 4 c h a r a c t e-r i z e d in that the selective capturing probe is provided with at least one moiety of an affinity pair or at least one specific site whereto at least one moiety of an affinity pair can be attached.

6. A reagent combination for assaying nucleic acids c h a-r a c t e r i z e d in that it comprises:
(a) at least one modified primer of the target nucleic acid, provided with at least one detectable label or at least one specific site whereto at least one detectable label can be attached; and
(b) at least one capturing probe capable of selectively hybridizing with the target nucleic acid, provided with at least one moiety of an affinity pair or at least one specific site whereto at least one moiety of an affinity pair can be attached.

7. A reagent combination for assaying nucleic acids c h a- r a c t e r i z e d in that it comprises:
(a) at least one modified primer of the target nucleic acid, provided with at least one moiety of an affinity pair or at least one specific site whereto at least one moiety of an affinity pair can be attached; and
(b) at least one detector probe capable of selectively hybridizing with the target nucleic acid provided with at least one detectable label or at least one specific site whereto at least one detectable label can be attached.

8. A kit for assaying nucleic acids c h a r a c t e r i- z e d in that it comprises in packaged form a multicontainer unit having:
(a) at least one modified primer of the target nucleic acid, provided with at least one detectable label or at least one specific site whereto at least one detectable label can be attached;
(b) at least one capturing probe capable of selectively hybrizing with the target nucleic acid, provided with at least one moiety of an affinity pair or at least one specific site whereto at least one moiety of an affinity pair can be attached;
(c) optionally a container comprising at least one template dependent polymerization agent;
(d) optionally a container with the four deoxynucleoside triphosphates;
(e) optionally a facility for the polymerization and the hybridization process;
(f) optionally a facility for the separation of the copies of the target nucleic acid
(g) optionally a facility for assaying the label.

9. A kit for assaying nucleic acids c h a r a c t e r i- z e d in that it comprises in packaged form a multicontainer unit having:
(a) at least one modified primer of the target nucleic acid, provided with at least one moiety of an affinity pair or at

least one specific site whereto at least one moiety of an affinity pair can be attached;

(b) at least one detector probe capable of selectively hybridizing with the target nucleic acid, provided with at least one detectable label or at least one specific site whereto at least one detectable label can be attached;

(c) optionally a container comprising at least one template dependent polymerization agent;

(d) optionally a container with four nucleoside triphosphates;

(e) optionally a facility for the polymerization and the hybridization process;

(f) optionally a facility for the separation of the copies of target nucleic acid; and

(g) optionally a facility for assaying the label.

10. A method as claimed in claim 1 substantially as hereinbefore described with reference to any one of Examples 1 to 6 herein.

Published 1988 at The Patent Office, State House, 66/71 High Holborn, London WC1R 4TP. Further copies may be obtained from The Patent Office,